

	<p>Journal Homepage: -www.journalijar.com</p> <p>INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)</p> <p>Article DOI:10.21474/IJAR01/5188 DOI URL: http://dx.doi.org/10.21474/IJAR01/5188</p>	
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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF CELLULASE PRODUCING *MICROBACTERIUM BARKERI* ISOLATED FROM AGRICULTURAL WASTE.

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Manuscript Info

Manuscript History

Received: 16 June 2017
Final Accepted: 18 July 2017
Published: August 2017

Keywords:-

Cellulase, *Microbacterium barkeri*, agricultural wastes.

Abstract

The present investigation was undertaken to isolate and screen an efficient cellulase producing bacteria from agricultural waste. Bacterial cultures were isolated from agricultural wastes such as groundnut shell, coconut coir, vegetable peel etc. A total of 28 bacterial cultures were isolated on nutrient agar. The isolates were screened for cellulase production by plating on CMC and Congo red agar media to observe the zone of cellulose hydrolysis. Among all 28 tested bacterial strains, 12 strains showed cellulase production on CMC and congo red agar. Further these 12 isolates were used for crude enzyme production. The carboxy methyl cellulase (CMCase) activity was assayed using the method described by Miller, with some modifications. S7 bacterial isolate which showed maximum enzyme activity (0.430 IU/ml/min) was further subjected to biochemical and molecular characterization. On the basis of 16s rRNA molecular characterization, the isolate S7 was identified as *Microbacterium barkeri*. Therefore, the cellulase producing *Microbacterium* sp. isolated and identified in this study have prospective for further use and study, such as to isolate efficient or novel cellulases with unique characteristics and various applications.

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Introduction:-

Cellulose is the most abundant biomass on earth (Venkata et al., 2013). Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta et al., 2003). Presently huge amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar et al., 2011). It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, that are of great interest for utilizing cellulose as an energy resource. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel et al., 2006). Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere (Lynd et al., 2002). Increasing knowledge of the mode of action of cellulase; they are used in enzymatic hydrolysis of cellulosic substances (Kubicek et al., 1993). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not

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exploited or which could be used more efficiently (Sonia et al., 2013). Cellulases are used in the textile industry for cotton softening and denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications. Overall cellulase can be commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes will also grow rapidly (Cherry et al., 2003). Also commercial enzymes are expensive, because they are produced from refined substrates and usually patented organisms (Philip et.al, 2016), hence there is a need to isolate efficient strains. Thus, the aim of the study was to characterize the cellulase producing *Microbacterium sp.* isolated from agricultural waste.

Materials and Methods:-

Collection of samples:-

Agricultural wastes such as groundnut shell, coconut coir, vegetable peel etc were collected in sterilized polythene bags and were further processed within 24 hours of procurement.

Isolation and screening of cellulose degrading bacterial strains:-

Cellulose degrading bacteria were isolated from the collected samples. The samples were plated with sevenfold dilution on Nutrient agar plates by serial dilution method. The plates were incubated at 37°C for 48 h. The colonies obtained were maintained on Nutrient agar. The isolates were screened for cellulase production by plating on CMC and Congo red agar media to observe the zone of cellulose hydrolysis. After the allotted growth time, the CMC agar plates with the isolates were stained with Grams iodine solution to visualize the cellulase activity. This solution stains the agar containing CMC brownish purple and leaves areas without CMC clear, described here as halos. The appearance of clear halos around the colonies confirms cellulase activity by the bacteria. Each plate was flooded completely with approximately 5 ml of the Grams iodine solution using a Pasteur pipette. The plates were allowed to sit for 5 minutes until the dye settled into the media. More the zone of clearance around the colony more will be its efficacy for producing the enzyme cellulase (Maki et al., 2011).

Production of cellulase crude enzyme:-

Cellulase enzyme was produced using growth medium. The screened isolates were inoculated in the growth medium and kept in a shaker incubator at 200 rpm for 72 hr. Then the cell free extract was procured by centrifugation. The supernatant was considered as the crude enzyme.

Estimation of Cellulase enzyme:-

Estimation of Cellulase enzyme activity was assayed using Dinitrosalicylic acid (DNS) reagent (Miller, 1959) by estimation of reducing sugars released from CMC. Crude enzyme was added to 0.5 ml of 1% CMC in 0.05 M phosphate buffer and incubated at 50°C for 30 min. After incubation, the reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100°C in water bath for 10 minutes. Sugars liberated were determined by measuring absorbance at 540 nm (Patagundi et al., 2014). Cellulase production was estimated by using glucose calibration curve (Shoham et al., 1999). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1 mol of glucose per minute under standard assay conditions (Muhammad et al., 2012).

Characterization of selected isolate on the basis of morphology and biochemical tests:-

The bacterial isolate showing maximum enzyme activity was selected for characterization on the basis of morphology and biochemical tests. The parameters investigated were colony morphology, Gram's reactions, motility, carbohydrate fermentation, catalase production, oxidase test, MR-VP reaction, casein hydrolysis, pectin hydrolysis, starch hydrolysis, growth in 2% NaCl and oxygen requirement.

Molecular identification of the selected cellulolytic bacteria:-

The selected bacterial isolate which showed maximum cellulase activity was further subjected to molecular identification by 16s r RNA sequencing.

Isolation of genomic DNA:-

Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit. An isolated bacterial colony was picked and suspend in 1ml of sterile water in a microfuge tube. It was centrifuged for 1 minute at 10,000–12,000 rpm to remove the supernatant. Then 200 µl of Insta Gene matrix was added to the pellet and incubate at 56°C for 15 minutes. Further it was Vortex at high speed for 10 seconds and the tube was placed in a 100°C in heat block or boiling water bath for 8 minutes. Finally, the content was vortexed at high speed for 10

seconds and spun at 10,000 - 12,000 rpm for 2 minutes. In result, 20µl of the supernatant was used per 50 µl PCR reaction.

Identification of bacteria by sequencing of the 16s rRNA (PCR Protocol):-

The 16s rRNA Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler

Primer Details:-

1µL of template DNA was added in 20 µL of PCR reaction solution. 27F/1492R primers was used for bacteria and then PCR reaction was performed under following conditions: Initial Denaturation 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec. Final Extension at 72°C for 10 min. DNA fragments were amplified about 1,400bp in the case of bacteria. A positive control (*E.coli* genomic DNA) and a negative control in the PCR were included.

Purification of PCR products:-

Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTGTTACGACTT	22

Sequencing protocol: Single-pass sequencing was performed on each template using 16s rRNA universal primer given below. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequencing Primer Details:

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

Sequence data was aligned and analyzed for Identifying the bacterial sample.

Bioinformatics protocol and Construction of phylogenetic tree:-

The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of the obtained sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering. (Dereeper et al., 2008).

Results:-

A Total of 28 bacterial cultures were isolated on nutrient agar. The isolates were screened for cellulase production by plating on CMC and Congo red agar media to observe the zone of cellulose hydrolysis. Among all 28 tested bacterial strains 12 strains showed cellulase production on CMC and congo red agar. Further these 12 isolates were used for crude enzyme production. The carboxy methyl cellulase (CMCase) activity was assayed using a method described by Miller, with some modifications. S7 bacterial isolate showed maximum enzyme activity (0.430 IU/ml/min). S7 was further subjected to biochemical characterization. The results of the physico-biochemical characteristic of S7 has been tabulated in table 1.

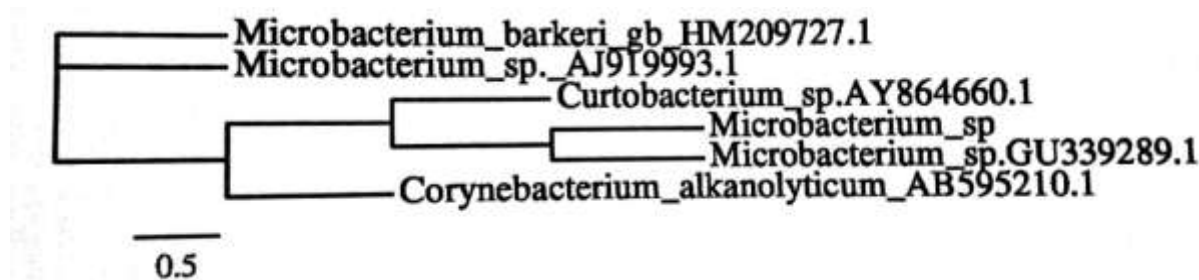
Table 1:- Physio-biochemical characteristics of bacterial isolate S7

Gram staining	Gram +ve
Cell shape	Rods
Colony color	Pale yellow
Motility	Non motile
Catalase	+
Oxidase	-
Methyl Red	+
Voges-Proskauer	-
glucose	+
Oxygen requirement	+
Casein hydrolysis	-
Starch hydrolysis	+
Pectin hydrolysis	+
Growth in 2% NaCl	+

The highest cellulolytic enzyme producing strains S7 was further subjected to 16s rRNA sequencing. On the basis of molecular characterization the isolate S7 was identified as *Microbacterium barkeri*. The Contig Summary and phylogenetic tree of *Microbacterium barkeri* obtained has been shown in fig 1. and fig 2. respectively.

>Contig

CTCTTACACATGCAGTCGAACGATGAAGCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAGTAA
CACGTGAGCAACCTGCCCCCTGACTCTGGGATAACAGCCGGAACGGTTGCTAATACCGGATATGCATC
ATGGCCGCATGGTCTGTGGTGGGAAAGATTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTT
GGTGAGGTAGTGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGG
ACTGAGACACGGCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCT
GATGCAGCAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGC
GAAAGTGACGGTACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
GCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTGCGCTCTGCTGTGAAA
ACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAA
TTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGAGGAACACCGATGGCGAAAGCAGATCTCTGGGC
CGTTACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGCTTAGATACCCTGGTAGTCCACCCCG
TAAACGTTGGGAACTAGTTGTGGGGGCCTTTCCACGGTCTCCGTGACGCAGCTAACGCATTAAGTTCC
CCGCCCTGGGGAGTACGCCGCACGCTAAACTCAAAGGAAATGACTGGGGACCCGCACAGCGCGGAGC
ATGCGATAATCGATGCACGCGAGAGCTACCAATGCTGGACATACACCGGAACGTCTGTAACAGTCGCC
CCCTTTTGTCTGTACAGTGTTCATGCTGTCTGCTCAGCTCTGCCTGGAATGTGGGTAGTACAGCACGAC
CAAGCATCGTCCATGTGCAGCAGCTATGTGTACTCATGCATCTGCATGTCATCGACGACGTAGATAGT
CGATCCATGACCTAGTCTGTGCCATGTCTCAATGCCGTACCAGTGTCAGCTGGTGAGCCAGTAGGCTTC
AGCA

Fig.1:-Contig Summary of *Microbacterium barkeri***Fig.2:-**Phylogenetic tree of *Microbacterium barkeri*

Discussion:-

The ability to degrade cellulose is a character distributed among a wide variety of bacteria. Studies on cellulolytic activity has isolated various bacteria from different environmental sources. (Hatami et al., 2008). Several

microorganisms have been discovered which have capacity to convert cellulose into simple sugars (Perez et al., 2002). Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose (Aristidou and Penttila, 2000).

Evaluating cellulase production between isolates can be a challenge because bacteria produce multiple types of cellulases (endoglucanase, exoglucanase, and β -glucosidase), which can be found to exist as free extracellular enzymes as well as found in enzyme complexes or cellulosomes expressed on the cell membrane (Maki et al., 2009). Thus, we initially use qualitative tests such as the CMC test. CMC agar allows us to identify isolates with cellulase activity on soluble cellulose such as CMC thus representing mainly endoglucanase and β -glucosidase activities (Kasana et al., 2008). All isolates may vary in growth properties which would not allow us to easily compare and quantify cellulase activities of all cellulase positive isolates in an equal manner. Additionally, some bacteria grow more rapidly than others, while still; cellulase production may be induced by different substrates for varying species. Thus, using qualitative screening methods is essential to narrow down isolates which may be more unique for further cellulase study in the future.

Furthermore, advanced studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation. Species-specific DNA sequences can be used for the identification of bacterial species. The 16S-23S rRNA has proven useful for identification of strains and species (Gurtler and Stanisich, 1996). Several strains of *Paenibacillus*, *Bacillus*, *Microbacterium* and *Streptomyces* have been found to produce cellulases and their cellulases have been well studied; these strains represent important cellulase degrading genera (Maki et al., 2011). Uniquely *Microbacterium* has been isolated from the gut of termites (Wenzel et al 2002). Some *Microbacterium* sp. exhibit particularly high filter paper activity and xylanase activity when a consortium of aerobic cellulase producing bacteria were studied (Okeke and Lu, 2010)

Therefore, the cellulase producing *Microbacterium* sp. isolated and identified in this study have prospective for further use and study, such to isolate efficient or novel cellulases with unique characteristics, or potential to use the strain along with others to create microbial consortia with a high efficiency for degrading complex cellulose containing biomass such as lignocellulose. According to this study, the isolation, screening and identification methods were efficient for allowing identification of capable cellulase producing bacteria. Finding naturally occurring cellulase degrading bacteria from the environment is important to help overcome costly hurdles in the industrial processes. Optimization of different physico-chemical parameter of the production medium is required to get the maximum yield of the enzyme so that the cellulase producing isolates may be useful in developing good cellulases or produce efficient cellulase producing systems such as microbial consortia which can be used for various industrial applications like decomposition of wastes and residues Sewage treatments, biowashing, bioethanol production etc. Thus, isolation, screening and characterization may act as a foundation for the discovery of such beneficial enzymes.

Acknowledgements:-

The authors acknowledge the immense help received from the scholars whose articles are cited and included in references of this paper. The authors are grateful to all authors/ editors/ publishers of all those articles, papers, journals and books from where the literature for this article has been reviewed and discussed.

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